Clean Version of Substitute Specification
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Biological joint construct

The present invention relates to a biological joint construct and processes for its production, and to bone tissue that contains transfected cells and processes for its preparation.

To date, joint defects can only be treated surgically by joint resection, joint reinforcement, alloplastic replacement with plastic or metal implants or material combinations thereof, or by biological implants. Alloplastic implants, however, are not integrated and loosen under loading. As a biological implant, only foreign tissue, for example knee joints, can be used as 'allotransplants'. Foreign tissue transplants require life-long immunosuppression with the danger of soft tissue tumor formation. An autogenous biological replacement can so far only be carried out partly by means of individual components. In addition to the known surgical measures, scientific approaches at present comprise the reconstruction of small cartilage defects by cartilage cell transplantation using a periosteal flap (Brittberg method). There have been various approaches to reconstruct individual components:

US patent 5,053,050 describes compositions for the repair of cartilage or bone, cartilage or bone cells being incorporated into a biological, resorbable carrier substance which contains serum, fibrinogen and thrombin. US application 5,041,138 describes a process for the production of a cartilaginous structure by population of a carrier substance with cartilage cells. In a similar manner, US application 5,786,217 describes a process for the production of a multicellular cartilaginous construct, in which cartilage precursor cells are applied to a carrier material and differentiate by means of further culturing and form cartilaginous substance. US patent 5,736,732 describes a polymeric carrier substance which contains chondrocytes and is suitable for forming cartilaginous structures in vivo. WO 98/42389 discloses a biological material for the treatment of bone or cartilage defects comprising periosteum and bone- or cartilage-forming cells. WO

97/46665 discloses an implant comprising a cartilaginous layer grown in vivo, which is combined with a bone replacement material. WO 99/25396 discloses a mixed tissue comprising a mixture of dissociated chondrocytes and osteoblasts in and on a polymeric matrix. A hybrid tissue is thus disclosed which is neither an osseous tissue nor a cartilaginous tissue. WO 96/03160 discloses a suspension comprising, inter alia, chondrocytes and osteocytes and fibrinogen, which are reacted with a thrombin solution to give a cell-fibrin matrix. EP 0 339 607 discloses a composition which can contain chondrocytes or osteoblasts or other cells in fibrin adhesive. DE 195 43 110 discloses a sterile bone material of native origin for transplantation, which is essentially free of fat, connective tissue and cartilaginous matter and is obtainable by dry heating and subsequent steam sterilization. The bone material can also be present in granular form. It is known that certain growth factors can stimulate the proliferation of cells of very different types. International application WO95/22611 describes processes and compositions for gene transfer in bone cells in vivo, in particular for the gene transfer of osteotropic genes, which can stimulate the growth of bone precursor cells in vivo.

To date, only individual components have thus been reconstructed successfully, for example bone or cartilage. Biological individual components can reconstruct complex, mostly osteochondral defects, but not adequately. The reconstruction of complex joint structures or complex biological joint replacement with osseous, cartilaginous, capsular and ligamentous components is still unsolved.

It is an object of the present invention to make available an advantageous biological joint construct.

This object has been achieved by means of the biological joint construct, which is at least partly prepared in vitro, according to the invention. In the context of this application, "in vitro" means that a process takes place outside the human or animal body.

This also includes "ex vivo" manipulation of cells, that is the culturing of isolated cells, their proliferation and modification. However, it does not involve a joint construct grown naturally in the human or animal body. The joint construct according to the invention comprises at least one biocompatible carrier material, cartilaginous tissue comprising chondrocytes and/or chondroblasts and cartilaginous substance, osseous tissue comprising osteoblasts and/or osteocytes and bone substance, cartilaginous and osseous tissue being firmly connected to one another.

Carrier materials are materials which have no cytotoxic effects, which make possible the accumulation of cells and allow the proliferation and differentiation of the cells to give tissue-synthesizing cells. The material should furthermore have a stable, physiological pH. To analyze these criteria, the following investigations can be carried out:

By means of electron microscopy, the surface topography of the material can be analyzed and the accumulation of the cells checked. Metabolic tests, for example the XTT test (cell proliferation kit, obtainable from Roche Diagnostics, Mannheim) produce a conclusion about the proliferation of the cells by measurement of their metabolic activity. What is involved here is a proliferation test for living cells, in which a sodium tetrazolium carboxanilite, in short a tetrazolium salt (having the abbreviation XTT) is added to the medium. XTT corresponds to sodium tetrazolium carboxanilite. A cytotoxic effect can thus be excluded. The tissue-typical matrices of cartilage, bone or capsule can be detected by means of histological methods. The tissue-typical matrix can also be determined by immunohistochemical procedures.

For cartilaginous tissue, spongy webs, viscous, gelling and solidifying gels or net-like filamentous tissue are employed as carrier substance. Biological variants here are fibrin-thrombin complexes, collagen gels or alginates. Conceivable synthetic materials are collagen, hydrogels or viscous polymers. If the web-

like carrier materials are used for cartilage, collagen webs and, as synthetic variants, polylactide, polyglycoside or polyurethane, can be used.

The carrier materials for bone as a rule have a solid, shapeable or processable, three-dimensional porous structure (porosity about 80-90%), it being possible for the pores to be connected to one another. The surface topography should be slightly roughened. Carrier substances can essentially consist of collagen, calcium phosphate or fibrin. However, they can also consist of synthetic polymers. As bone carrier materials, the following stable, three-dimensional, porous materials can be used: human or animal spongy bone, sintered bone, coral matrix, demineralized bone matrix (biological variants), calcium phosphate compounds, polylactide, polyglycolide, and other polymers (synthetic variants) can be used. As viscous, gelling and solidifying gels, the following materials can be employed: fibrin-thrombin complexes, collagen gels, alginates (biological variants), hydrogels, viscous polymers (synthetic variants). The bone carrier materials can furthermore be coated with adhesive molecules which facilitate the attachment of cells. Such adhesive molecules are preferably fibronectin or laminin. It is essential that no rejection reactions to the carrier materials occur in the recipient's body.

Ligamentous tissue networks or webs are suitable for capsular components. Carrier materials which are generally employable for the preparation of ligamentous components are generally membranous synthetic resorbable fiber materials, for example polyglactin, polyglycolic acid, polylactic acid or hyaluronic acid.

The carrier materials for ligamentous components can also be used as carrier materials for capsular components.

A further aspect of the present invention is the use of spongiosa as a carrier material of the ligamentous component. Surprisingly, it has been found that spongiosa is very highly suitable for stimulating bone of precursor cells to the synthesis of osseous

substance. Preferably, in this case this is autoclaved, human, allogenic spongiosa, which is obtained from femur heads. Most preferably, the material is obtained from femur heads which are cleaned of loose stroma by means of a water jet and ultrasonic treatment. Autoclaving is then carried out at 134°C and 2.5 bar for sterilization. The material is sterile, nonimmunogenic, resorbable and consists of hydroxyapatite and denatured type 1 collagen.

The cartilaginous tissue contained in the joint construct according to the invention contains chondrocytes and/or chondroblasts and cartilaginous substance. Cartilaginous tissue is understood as meaning viscoelastic, avascular and uninnervated supportive tissue formed from cartilage cells (chondrocytes) and - various - parent substance and various types of fiber. Cartilaginous substance is understood as meaning the parent substance together with the various types of fiber. The parent substance contains glycosaminoglycans and proteoglycans, preferably hyaluronic acid, chondroitin sulfate and keratan sulfate. Proteins and mineral constituents are furthermore present. As types of fiber, elastic fibers, collagen fibrils or collagen fibers can occur.

Bone tissue is to be understood as meaning a tissue which consists of bone cells, collagen fibers and a calcified parent substance. Bone substance within the meaning of the application comprises collagen, glycosaminoglycans and proteoglycans, and also inorganic substances, mainly calcium phosphate, which occurs in the form of hydroxylapatite crystals. To improve the new vessel formation, the osseous tissue can contain growth factor proteins or osteoblasts which have been transfected with genes coding for growth factors. Finally, tissue-forming cells can also be present, which promotes that blood vessel formation (e.g. endothelial cells or their precursor cells).

According to the invention, cartilaginous tissue and osseous tissue of the joint construct are firmly connected to one another. In this case, the carrier material of the bone is interlocked with

the new bone substance, it is also partially integrated into the cartilaginous component. There is thus no sharp separating surface between osseous and cartilaginous tissue, rather a solid compound is obtained by interlocking and interdigitation. Preferably, this is achieved by the osseous tissue having a rough or porous surface, into which the cartilaginous tissue can grow.

The bone and cartilage cells contained in the joint construct had been isolated according to customary methods before the production of the joint construct. It is also possible to isolate precursor cells, which only differentiate to give bone or cartilage cells during culture in vitro.

The biological joint construct can have any desired size. Thus the partial replacement of a joint surface, the total replacement of a joint surface or the replacement of a total joint having two joint surfaces is possible.

On partial replacement of a joint surface (without ligament replacement), an individual preformed construct or osteochondral cylinder can be used. When using an individual preformed construct, a specific cartilage/bone defect of a joint can be replaced by an individual osteochondral construct. The shape is in this case determined by the bone components. After cell obtainment and production of the components, the construct is preformed ex vivo, that is it is made-to-measure with the aid of individual data acquisition by computer tomography or magnetic resonance investigations on the defect to be corrected. The anchoring is then carried out by means of the bone pegs. To avoid a particular individual production of a construct such as described, uniform osteochondral cylinders can also be prepared, which are inserted into a defect in the sense of a 'mosaic plastic' and reconstruct the joint surface. These cylinders can be produced in various sizes and shapes. In each case, they consist of a cartilaginous layer and a bone peg. Both components are loaded with the respective types of cell. The bone cells can also be transfected with growth factors in order additionally to initiate the bone

formation and vessel formation. Preferably, the cylinders have a height of 5 to 25 mm and a diameter of 2.5 to 15 mm. The layer thickness of the cartilage is preferably 0.5 to 2 mm, that of the bone will customarily vary between 3.5 and 23 mm. Possible conceivable shapes are cylinders having a round cross section, having a triangular cross section or having a polygonal cross section (e.g. penta-, hexa-, hepta- or octagonal). Likewise, parallelepipeds having a rectangular cross section (quadrangular) or having a square cross section are used. The cartilages are shaped such that their surface has a convex curvature or a plane surface without curvature. The use of osteochondral cylinders was tested in a rabbit model and was successful in the reconstruction of an osteochondral defect. The advantage of the method is the simpler production and the possible minimal-invasive use.

On total replacement of a joint surface, the joint construct preferably has an anatomically useful shape, for example it has one joint side whose surface consists of cartilaginous tissue and can have contact with another joint part, and it has an anchor side, which consists partly of osseous tissue and which can be used for anchoring the construct in a bone shaft. The anchor side is preferably designed in the form of a cylindrical peg, which can be particularly readily anchored in a bone shaft. The joint side particularly preferably has a concave or convex surface. However, other joint construct forms are also conceivable, depending on the joint, for whose repair the construct is to be used.

The joint constructs according to the invention can likewise have at least one cartilaginous component. The cartilaginous component consists, for example, of a stringy, fibrous or membranous biomaterial. It can be fixed to cartilage connection at sites of the bone component of the joint construct according to the invention. Possible fixing species are bioadhesive, for example by means of fiber-thrombin complexes, supportive, transossary suture or passage through the bone component in a bone channel. On total replacement of a joint surface, the production of the ligament structures is not absolutely necessary, since the capsule-ligament

structures present can be used in the defect. These are, as a rule, drilled channels, through which the suture material is passed and with which capsule-ligament structures can be fixed. Preferably, the cartilage-bone construct is employed without additional fixing of the capsule or ligaments.

A further embodiment of the present invention consists in a biological joint replacement which comprises at least two joint constructs. Two joint constructs can in this case have contact with one another with their cartilage-coated joint sides, while the anchor sides facing away from one another can be anchored in two different bone shafts. This joint replacement is preferably further stabilized by application of at least two ligament components. Finally, a biological joint replacement of this type can also have a joint capsule. To this end, a membranous biomaterial is fixed to capsule connection areas of the bone component either by bioadhesive or by suture. As a result of the embodiment described, the complete replacement of a joint having two joint surfaces can be carried out. The complete joint replacement having two joint components is especially necessary in arthrosis or arthritis and represents the most complicated form of joint replacement. In principle, two joint parts consisting of cartilaginous and bone component are prepared. On one side, the joint surface is convex, on the other side, fitting this, concavely shaped, so that a proper articulation of the surfaces is possible on movement. The components are produced individually and can also be implanted individually, such as the total replacement of only one joint surface as described above.

Preferably, existing capsules and ligaments of the old joint are used in order to connect the individual parts and to stabilize the new joint. To this end, the ligaments are fixed to the bone component by means of sutures, which are drawn through drilled channels. The component can have a well in this position. Alternatively, the two joint parts are connected to one another by suture material until a neocapsule has formed in vivo after transplantation. If a ligament replacement by means of tissue

engineering is necessary, the ligament replacement can be produced as follows. Firstly, autologous fibroblasts from the dermis are isolated by means of a skin biopsy and proliferated in vitro. A ligamentous biomaterial is populated with a cell suspension (2.5 to 5 x 10^7 cells per ml of medium). Possible biomaterials are, for example, collagen webs and, acellular cutis, lyophilized dura or synthetic ligaments made of PGLA. The ligament construct is grown in vitro for 3 to 7 days. Finally, the structure is fixed to the bone component by means of suture or fibrin adhesive. The fixing is initially carried out here only on one joint side, the second connection is carried out only after implantation in the course of the operation.

Alternatively, the ligament structures can also be carried out by the transplantation of tendons or parts thereof, as is already the present state of the surgical art.

The present invention moreover relates to a process for the production of a biological joint construct. This process comprises the production of a bone component, the provision of a cartilage component, the connection of these two components, and the growth of the resulting construct in vitro.

The production of the bone component is carried out by population of a biocompatible carrier material with osteoblasts. Possible carrier materials are the above-mentioned carrier materials for bone. Precursor cells of osteoblasts can be obtained by small bone biopsies of the pelvis, sternum, of the skull and jaw and or of tubular bone. The loose stroma is rinsed out from the bone samples and, after centrifugation, plated out in a culture flask. The solid bone components can likewise be cultured, since further cells can be obtained therefrom by migration. The growing cells are split in the subconfluent stage and passaged two to three times. Alternatively to bone samples, aspirates of bone marrow from the pelvis or from the sternum can also be used. Here, the bone is punctured, for example, with a cannula, and the sample is obtained by aspiration. The aspirates are rinsed in heparin

medium, centrifuged through a density gradient in order to separate off red corpuscles, and finally plated out in a culture flask. The osteoblastic phenotype of the cells in culture can be checked by detection of the bone-specific proteins alkaline phosphatase and osteocalcin in the culture medium. Immunohistochemical stainings of control cultures are likewise conceivable. The blood supply of the biological joint replacement by newly formed vessels is of crucial importance for the survival of the cells and the healing of the construct. For this reason, the bone component can be modified in three ways: it can contain growth factor proteins which induce new formation of vessels (1), it can contain osteoblasts which are transfected with growth factor genes (2) or it can contain vessel-forming cells which lead to blood vessel formation (3).

Angiogenic growth factors can be stored directly in recombinant form in the viscous matrix of the bone component in efficacious concentration. Possible factors are vascular endothelial growth factor (VEGF) and isoforms thereof, basic fibroblast growth factor (bFGF), angiopoietin 1 and 2 (Ang I and II). The concentration of the factors is customarily 0.1 to 10 ng/ml of viscous matrix. The growth factor(s) can advantageously be added to a calcium chloride/thrombin solution, which is then added to an osteoblast suspension in fibrinogen solution. A three-dimensional fibrin network with adhering osteoblasts is thereby formed in the culture medium and stored growth factor.

The transfection of osteoblasts with vessel-forming growth factors is described in detail in a later part of the application text.

Vessel-forming cells, "endothelial cells" or their precursor cells, can also be incorporated into the viscous matrix of the bone component, mixed with the osteoblasts. Microvascular endothelial cells are isolated from the dermis of a patient according to a protocol known per se and proliferated in vitro using endothelial cell medium (established technique). An osteoblast-fibrinogen suspension is prepared, the medium used

being "endothelial cell medium", in which osteoblasts can grow readily. The mixed cell osteoblast-endothelial cell-fibrinogen suspension is then absorbed in or applied to the porous, solid carrier matrix of the bone component.

The three strategies described can also be combined. For example, an osteoblast-endothelial cell-fibrinogen suspension can be employed together with osteoblasts which have been transfected with growth factor genes. Just as osteoblast-endothelial cell-fibrinogen suspensions can be used together with recombinant growth factors.

Preferably, the bone carrier material is populated by a bone cell suspension in an aspiration chamber. Customarily, the bone carrier material is formed before population by bone cells in an anatomically useful manner. For example, the shaping leads to the formation of a joint side, which is to be used for the absorption of cartilaginous surface and for the formation of an anchor side, which is to be used for connection with a bone shaft.

Chondrocytes are preferably obtained from biopsies of cartilaginous tissue of the ear, the rib and the joints by enzymatic dissolution. By inoculation of biomaterials such as collagen sponges and fibrin adhesive, the cells can be further proliferated organotypically in a three-dimensional culture. By this means, it is also avoided that the cells dedifferentiate to fibroblasts. The chondroblastic phenotype is retained. For the preparation of the cartilaginous component, a suspension of chondrocytes can now be prepared either in a liquid medium or in a gelatinous material. For example, cartilaginous cells can be suspended in the thrombin component of a fibrin adhesive and, after mixing with the fibronectin components, poured into a specific previously prepared culture form. A solid structure thereby results, which is coated with culture medium and is cultured in an incubator. Alternatively, biocompatible carrier substances can also be populated by cartilaginous cells. For example, sponges made of collagen are inoculated with

chondrocytes. The inoculated collagen webs can be cultured in an incubator. In a preferred embodiment of the invention, bone and cartilaginous component are prepared separately of one another and cultured separately. After the respective cells have formed adequate tissue, bone and cartilaginous component are combined with one another by means of fibrin adhesion. It is essential to the invention that during the connection of bone and cartilaginous component, the carrier material of the bone component is integrated into the cartilage. The resulting construct can now be cultured in vitro, such that the cells are stimulated to adhesion and to the synthesis of their tissue-specific extracellular matrix, on account of this a biological cross-linkage of the combined components is achieved.

In a particularly preferred embodiment of the invention, bone and cartilaginous component are not prepared and cultured separately. Rather, during the setting of the fibrin adhesive in the preparation of the cartilaginous component, the carrier material of the bone component, which has still not been populated by osteoblasts, is forced into the cartilaginous layer, such that it is firmly bound. Population by osteoblasts takes place only later.

A further embodiment of the invention also comprises the preparation of a ligament component made of fibrous materials and fibroblasts. In the course of this, fibroblasts can be inoculated from suspension onto ligamentous materials and cultured in an incubator. Similarly to this, embodiments can also include the production of a capsular component from fibrous, membranous materials and fibroblasts.

Furthermore, the process according to the invention can include the attachment of the ligament binding sites or capsule binding areas to the carrier material of the bone component. Joint ligaments or capsular components can be fixed thereto. Ligament constructs are preferably connected thereby to the bone component in that the ligament construct is drawn through a drilled channel in the bone component.

A further aspect of the invention is a process for the production of bone tissue, according to which bone or bone precursor cells are isolated, these cells are transfected by nonviral gene transfer with a growth factor and are used for the population of a biocompatible carrier material. The resulting construct is finally cultured further in vitro. Optionally, the isolated cells are proliferated in vitro before the transfection. The tranfection can be stable, but is preferably a transient transfection. During the transient transfection, the cells are only temporarily, i.e. not permanently, genetically modified by the inclusion of DNA. The transfection can be carried out by various methods. In particle-mediated gene transfer, cells are fired on with plasmid- or naked DNA-coated gold particles by a "gene gun". Alternatively to this, the transfection can be carried out by electroporation. The preferred transfection method, however, is lipofection. The reagents TRANSFECTAM® (obtainable from Promega), FUGENE® (obtainable from Roche Diagnostics),LIPOFECTAMINE 2000®, LIPOFECTIN® (Life Technology) and ESCORT® (obtainable from Sigma) are preferably used here.

According to the invention, the cells are transfected with at least one growth factor gene. A growth factor or cytokine within the meaning of the invention is a compound which can stimulate the proliferation and/or differentiation of cells. Most growth factors are proteins or peptides, so that genes which code for these proteins or peptides can be transfected. Preferably, these genes are incorporated into plasmids which contain an additional regulatory and control sequence in order to guarantee the expression of the gene. These additional sequences include a replication origin, a promoter sequence, most preferably derived from the cytomegalovirus (CMV), optionally a Kozac sequence (a DNA sequence located close to the promoter, which should increase the expression), a polyadenylation signal, if appropriate a gene which mediates resistance to an antibiotic. According to the invention, it is also possible to transfect nucleic acids which only code for

one fragment of a growth factor, but which has the activity of a growth factor.

Possible growth factors whose genes can be transfected are "basic fibroblast growth factor" (bFGF), "insuline-like growth factor" (IGF I, II), "platelet derived growth factor" (PDGF-AA, -AB, -BB), "bone morphogenetic proteins" (BMP-1 to BMP-20), "vascular endothelial growth factor" (VEGF), factor XIII (F XIII), "transforming growth factor β" (TGF-β), angipoietin (Ang I, II) and other osteotropic factors. Preferably, however, the gene is transfected which codes for epidermal growth factor (EGF).

In a particular embodiment of the invention, the biocompatible carrier material is not only populated with transfected cells, but also with transfected cells in a ratio of 1:3 to 1:9 (transfected to nontransfected cells). The isolated cells can be various bone cells or bone precursor cells, but they are preferably stromal cells.

The invention moreover relates to bone tissue which contains at least one biocompatible carrier material and osteoblasts, which have been transfected in vitro by nonviral gene transfer with a gene which codes for a growth factor. Preferred embodiments relate to bone tissue which has been prepared with the aid of transfected cells according to one of the processes for the production of bone tissue described above.

The bone tissue according to the invention has two significant advantages.

On the one hand, nontransfected cells are stimulated to proliferation paracrinally by the secreted growth factors. In this process, the growth factor is produced by a cell and acts on another cell. This not only results in an improved population of the carrier materials during the in vitro phase, but also an improved binding of the tissue after implantation. The bone tissue is then available in vitro to growth factors, i.e. is a "cellular

growth factor supply system". The disadvantages known from other processes do not occur here: no cells are transfected apart from the desired ones. They are no risks to be feared which accompany the viral methods. Doses of recombinant growth factor protein need not be administered repeatedly, since secretion takes place "physiologically". On direct administration of growth factor, the risks of biological inactivity or lack of purity of the protein also exist.

The second significant advantage consists in the fact that certain growth factors can induce formation of vessels after implantation of the tissue. The new formation of vessels is one of the crucial problems in "tissue engineering". The factors can thus lead to an improved vascularization of the construct, which is a significant advantage for the properties of the implanted tissue.

Figure 1a) shows the fusion of the cartilaginous component with the carrier material of the bone. During the solidification process of the cartilaginous component, the carrier material of the bone component is pressed into the cartilage construct.

Figure 1b) shows the attachment of the ligament components. Biological ligament components can be integrated onto the ligament connection sites (in this case by transverse drawing-through). The construct has a joint side and an anchor side for incorporation into the bone.

Figure 1c) shows views of a cartilage-bone construct. The carrier material of the bone component has been populated with osteoblasts. The cartilage component is firmly connected to the bone component. The construct has an anatomically correct shape for joint replacement. It has a joint side and an anchor side.

Figure 1d) shows the integration of the biological joint construct into the bone. Production of a one-sided joint replacement by incorporation of a cartilage-bone construct without ligament connection to the bone.

Figure 1e) shows the integration of a complete joint replacement with ligament connection. A two-part joint replacement was employed with ligament connection to bone after removal of the original joint (here finger base joint). The biological joint replacement has the correct anatomical form.

Figure 1f) shows a histological scheme after fusion of the cartilage component with the bone carrier material. The carrier material of the bone component (here spongiosa) has been integrated into the cartilage construct during the solidification process of the cartilage cell suspension (here fibrin adhesive). A stable connection between cartilaginous layer and carrier material of the bone results.

Figure 1g) shows a histological scheme of a cartilage-bone construct after synthesis of bone substance by osteoblasts. After population of the construct shown in figure 1f) with osteoblasts, new bone substance was synthesized.

Figure 1h) shows a histological scheme of the interface between cartilage and bone. In the scheme, the enlargement shows the interface between the cartilage and bone component with the carrier material of the bone. The interlocking of the carrier material with the new bone substance and the crosslinking of the cartilage with the bone is seen. The carrier material is partially integrated into the cartilage component.

The invention is illustrated in more detail by the following examples, animal experiments having to confirm the feasibility of the present invention, since experiments on humans do not appear justifiable for ethical reasons. The present invention, however, preferably relates to biological joint constructs which contains autologous cells, i.e. cells originating from the recipient or allogenic cells, that is cells originating from another human.

Example 1: Isolation of chondroblasts

The isolation of chondroblasts from rabbits is firstly described. This method is transferable to humans in principle.

The rabbit is anesthetized by an injection. The hair is shaved in the operation area and the skin is disinfected and sterilely covered. An incision of about 2 cm is made over the cartilaginous section of the rib. The rib section is exposed, and approximately 1.5 cm of rib cartilage including the perichondrium are removed. After staunching blood and rinsing, the wound is closed by backstitch sutures. A spray dressing is then applied.

The autologous rib cartilage is added to sterile Ringer's solution (G. Ph. 7 solution, obtainable from Delta Pharma), which has been supplemented with 200 IU/ml of penicillin and 20 µg/ml of streptomycin. The cartilage can be stored in the solution for some hours at 4°C, but must be processed within 6-8 hours. The cartilage tissue is comminuted to a size of 1-2 mm under sterile conditions using a scalpel (20/22) and added to a large petri dish (130 x 15 mm). The amount of cartilage is weighed.

50 ml of 2 mg/ml collagenase are then added to the petri dish containing the pieces of cartilage in a commercially available standard medium, specially prepared for chondrocytes, namely HAM's F12 medium (Hepes modification, containing 100 IU/ml of penicillin, 100 µg/ml of streptomycin). Incubation in the cell culture incubator follows for 16-24 h.

The cartilage cell suspension with remaining pieces of cartilage is pipetted off, diluted with the same volume of HAM's F12 medium (Hepes modification), 100 IU/ml of penicillin, 100 µg/ml of streptomycin, ≈ 50 µg/ml of ascorbic acid, ≈10% (v/v) of autologous serum, 2 mM glutamine and thoroughly shaken on a shaker in order to separate the persistent pieces of cartilage. This step is best carried out in closed 50 ml centrifuge screw-cap vessels.

The cell suspension is filtered into 50 ml screw-cap vessels through a cell filter (about 25 mm per vessel) and made up to 50 ml with abovementioned HAM's F12 medium. The cells are washed 3-4 times with 30 ml of PBS by centrifugation (10 min, 500 g, 21°C). After the last washing step, the cells are taken up in 30 ml of medium.

In order to determine the cell count, 0.5 ml of a 0.4% Trypan Blue solution is added to a test tube. After adding 0.3 ml of HBSS (Hank's salt solution) and 0.2 ml of cell suspension, the batch is carefully mixed and incubated at room temperature for 15 minutes. The cell count is then determined by counting with a hemocytometer.

Example 2: Isolation of osteoblasts

Various methods are available, firstly open bone biopsy (as migration or stromal cell culture) and secondly aspiration from bone marrow.

a) Bone marrow biopsy

After local anesthesia and a small skin incision, a sterile bone biopsy is taken by means of a well borer. Blocks of spongiosa of 0.5 to 1 cm^3 are bored out. The wound is then closed. Alternatively to this, 15 ml of bone marrow can also be aspirated. The spongiosa should be reused very quickly, if possible stored at 4°C for not longer than 12 hours in the transport vessel. The medium is discarded, the spongiosa are added to the petri dish and comminuted there into 2 to 3 mm small particles ("chips").

Migration culture:

3 to 4 particles are distributed into a well of a tissue culture plate having 6 wells and made up with 3 ml of medium, or 6 to 7 particles per 25 cm^2 growth area with 7 ml of medium. Incubation is carried out in an incubator at 37°C and 5% CO_2. The medium should be changed twice per week, in the course of this the cells are inspected under a phase-contrast microscope. After 5 to 9 days, the first cells are to be discerned, after 10 to 14 days a subconfluent cell lawn having a 65 to 75% bottom surface covering.

Stromal cell culture:

Firstly, the bone is freed from residues of muscle and connective tissue. With the aid of scissors and forceps, the spongiosa is comminuted into pieces which are as small as possible. The spongiosa fragments can be added to a 50 mm centrifuge tube with a screw closure and weighed therein. If the material contains much red bone marrow, a 75 cm^2 tissue culture flask can later be charged per 4 to 6 g of spongiosa. About 25 ml of medium 1 (basal medium

with antibiotics, e.g. medium 199, Gibco, 100 IU/ml of penicillin, 100 µg/ml of streptomycin) are now added to the comminuted spongiosa in the 50 ml centrifuge tube with screw closure and the cells are dissolved out by vortexing (high-frequency shaking, about 30 seconds, highest step). The supernatant is transferred to other screw vessels. This step is repeated until the medium is no longer turbid after vortexing. Finally, a trypsin (and/or collagenase) treatment (about 10 min, 37°C) can additionally be carried out in order to obtain further cells. The cell suspensions obtained are centrifuged at 250 g and 4°C for 10 min. The supernatants are discarded, and the cell pellets are resuspended in medium and distributed in culture flasks. The rinsed pieces of bone can optionally be used in a separate culture flask for the growth of residual cells (after trypsinization, however, these are to be rinsed well with medium).

In order to determine the cell count, after the resuspension the cell pellets are transferred to 50 µl of the suspension in an Eppendorf vessel and mixed with 46 µl of Trypan Blue. The erythrocytes are lyzed by addition of 4 µl of acetic acid. The nucleus-containing cells are counted in a Neubauer cell chamber (glass chamber for counting cells under a microscope). Approximately 10^7 cells of the cell suspension are then distributed per 75 cm^2 of culture flask. The change of medium is carried out after about 5 days, later twice per week. Adherent cells are first discernible on the second day, colonies from day 4 to 5. Subconfluence is achieved between day 9 and 11.

b) Isolation and selection of the bone precursor cells from bone marrow aspirate

After local anesthesia, a sterile bone marrow puncture is carried out. By aspiration with a large-volume cannula and a heparin-wetted syringe, approximately 15 to 20 ml of blood are taken from the posterior iliac crest. The sample is centrifuged at approximately 400 g, the supernatant is removed and the cell pellet is suspended in 5 ml of serum-free medium 1. A density

gradient centrifugation is then carried out through a 70% Percoll gradient (Percoll is the commercial name of a viscous solution having an increased density compared to water) or a Ficoll pad (Ficoll is a viscous solution having a higher density than water, the chemical composition differs from Percoll, but it acts according to the same principle. Both solutions are used for density centrifugation.) at 400 g for 30 minutes at 4°C in order to separate the red blood corpuscles from the mesenchymal cells. Finally, the mesenchymal cells are plated out and proliferated in a tissue culture flask.

The osteoblastic phenotype of the cells can be demonstrated by detection of the bone-specific proteins alkaline phosphatase and osteocalcin in the culture medium or by immunohistochemical staining of control cultures.

Determination of the alkaline phosphatase activity

The cells are washed twice with PBS. 2 ml of alkaline phosphatase substrate buffer (ROTIPURAN®, Roth, 50 mM glycine, 1 mM $MgCl_2$, 5 mM p-nitrophenyl phosphate, pH 10.5) are added per batch of a tissue culture dish having six wells. Incubation is carried out at 37°C for 15 minutes. The incubation time can be varied from 5 to 20 minutes. The incubated buffer solution is then mixed 1:1 with 1 M NaOH in a cuvette. Finally, the absorption is determined at 405 nm. The enzyme activity is indicated as absorption/cell count.

Osteocalcin staining

After removal of the medium, the cells are washed once with PBS. The cells are then fixed for 15 minutes with the solution A of the FIX & PERM kit (commercial name of a kit with which cells can be fixed and the membrane permeabilized so that stains and proteins can penetrate into the interior of the cell. Cell Permeabilization Kit, AN DER GRUB, obtainable from Dianova), the solution is then filtered off with suction. Solution B of the FIX & PERM kit is pippetted, protected from light, onto the monolayer together with the primary antibody (rabbit anti-human osteocalcin, PAESL + LOREI; diluted 1:25 in PBS) in the ratio 1:1 for 2.5 hours, rinsing is then carried out two to three times with PBS. Incubation with the secondary antibody (FITC-conjugated goat and rabbit IgG, Sigma, 1:80 in PBS) is carried out protected from light for one hour. According to the keyhole principle, the primary antibody recognizes a specific epitope. The secondary antibody then recognizes specific domains of the bound primary antibody. Following this, rinsing is carried out two to three times with PBS. The cells are then immediately investigated with an immunofluorescence microscope with FITC excitation switched on.

Alkaline phosphatase staining

For staining, a kit from Sigma Diagnostics, catalog No. 86-R was used. The following solutions are firstly prepared: the fixer

solution is formed by mixing 65 ml of acetone, 25 ml of citrate solution and 8 ml of 37% formaldehyde. The solution can be kept in a glass flask at 4°C for up to four weeks. The diazonium salt solution is prepared by mixing, in each case, 1 ml of FRV alkaline solution and sodium nitrite solution, incubation at room temperature for 2 minutes and addition of the mixture to 45 ml of H_2O. Finally, the alkaline dye mixture is prepared by addition of 1 ml of naphthol AS-BI alkaline solution to the above diazonium salt solution (protect from light). The fixer solution is brought to room temperature, then the cells are fixed for 30 seconds. The cells are then rinsed with H_2O for 45 seconds, care is to be taken that the cells do not dry out. Consequently, the cells are incubated for 15 minutes with alkaline dye mixture at room temperature in the dark. Rinsing is then carried out with H_2O for 2 min. The counterstaining is a carried out using hematoxylin solution. Hematoxylin is a dye which dyes tissue red in the microscopic investigation. Hematoxyline solution is added dropwise and left on the cells for 2 minutes, then it is rinsed well with water. The cells are dried in air and can be inspected under a light microscope.

Example 3: Isolation of fibroblasts

A piece of skin tissue is freed from fatty tissue and cut into pieces approximately 0.5 cm^2 in size. The pieces of skin are incubated in 2.5 U/ml of dispase either at 37°C for 3 h or at 4°C for 16 h. The epidermis is then removed and discarded. The pieces are comminuted as well as possible using a scalpel. The resulting paste is then treated with an enzyme solution, comprising about 90 U/ml of collagenase and about 140 U/ml of hyaluronidase and shaken at 37°C for 3 h. The paste is then centrifuged at 300 g for 30 minutes at 4°C, the supernatant is discarded, the sediment is taken up in 5 ml of medium 2 (medium 1, 10% (v/v) of serum; in the case of therapeutic use autologous, human serum is used, in the case of experiments fetal calf serum, obtainable from Gibco) and added to a 25 cm^2 tissue culture flask.

Should many undigested pieces still be present, instead of in medium, the sediment is taken up in 5 ml of about 185 U of collagenase/ml of buffer solution, added to a 25 cm^2 culture flask and incubated overnight in a cell culture incubator. On the next day, the collagenase-containing suspension is removed, centrifuged, the sediment is taken up in medium 2 and returned to the same flask.

Example 4: Preparation of the cartilage component

A) Collagen sponges

The cell suspension is adjusted to approximately 3 x 10^7 cells/ml. Collagen webs are cut into pieces approximately 1 x 1 cm in size (sterile). The webs are inoculated using an insulin syringe. 2 ml of cell suspension suffice here for 8 to 10 pieces of collagen. After 3 hours in the incubator, the pieces are distributed on a tissue culture plate having six wells and made up with, in each case, 8 ml of nutrient medium. The medium is changed every two days.

Animal experiment:

Primary bovine chondrocytes were inoculated as a suspension into sponges of bovine collagen type 1. The constructs were cultured in an incubator at 37°C and 5% CO_2 for seven days and then nude mice were subcutaneously implanted. Control groups were collagen sponges without cells and chondrocyte suspensions without carrier material. The explantation was carried out after three, six and nine weeks. The constructs were investigated histologically/immunohistochemically. The biomechanical testing was carried out by means of a "confined compression test" for the determination of the hydraulic permeability and of the compression modulus (biomechanical test procedure in which defined, cyclic pressures are exerted on a sample to be tested and force relaxation measurements are carried out). As a result, morphologically hyaline cartilage with immunohistochemical

detection of cartilage-typical collagen type 2 was seen. In the controls, the empty collagen sponge was resorbed for three weeks. The cell suspension developed only small pieces of cartilage. The rigidity of the constructs increased in the course of time (1.99 Mpa in week 9), but was reduced compared with native cartilage (5.25 Mpa). The hydraulic permeability showed significantly higher values than the control ($2.69 \cdot x\ 10^{-14}$ compared with 3.0×10^{-15} m^4/Ns).

b) Fibrin suspension

The cartilage cells are dissolved and, after centrifugation, introduced into the thrombin component of a two-component fibrin adhesive in a density of $2\text{-}3 \times 10^7$ cells/ml. The cell-containing thrombin component is then mixed with the fibrinogen component by synchronous syringing by means of a double syringe and poured into a previously prepared culture mold. A solid "clot" is formed by precipitation of the fibrinogen to give fibrin. Finally, it is covered with a layer of HAM's F12 medium (Hepes modification, addition of Hepes buffer solution to a nutrient medium) and incubated in an incubator at 37°C and 5% CO_2.

Animal experiment:

Primary chondrocyte cultures are established from rib cartilage biopsies and suspended at a density of 2×10^7 cells/ml in the thrombin component of a fibrin adhesive. The fibrinogen component is then mixed together by means of a double syringe with the chondroblast-containing thrombin component (4 IU of thrombin/ml) and poured into a structured, anatomically suitable mold. The fibrin adhesive solidifies as a function of the thrombin concentration within 5-10 minutes in the desired shape.

Animal experiment:

The constructs were implanted subcutaneously into nude mice. "Fibrin clots" without cells and chondrocyte suspensions without

fibrin were implanted into control groups. The explantation was carried out after three, six and nine weeks. The constructs were investigated histologically/immunohistochemically. The biomechanical testing was carried out by means of a "confined compression test" for the determination of the hydraulic permeability and of the compression modulus.

As the result, newly formed cartilaginous tissue was found microscopically in the group with cartilage cells in fibrin adhesive and no cartilage tissue was found in the control groups. Histologically, the cartilage constructs showed the morphology of hyaline cartilage with immunochemical detection of type 2 collagen. In the biomechanical testing, the cartilage constructs showed a rigidity (compression modulus) in the "confined compression test" of 0.59 Mpa and a hydraulic permeability of 1.03 x 10^{-14} m^4/Ns.

Example 5: Preparation of the bone component

A) Preparation of the cell-biomaterial constructs

The carrier materials are hydrated in culture medium one week before population with cells in order to achieve rehydration and pH stability.

By means of trypsinization, the cells grown as a monolayer culture are detached from the culture dish and, after centrifugation and counting, suspended in a small volume as an individual cell suspension. The medium is removed, one part is poured into a 1.8 ml cryo tube and frozen, and the rest is discarded. Osteocalcin and alkaline phosphatase is determined from the supernatants by means of an enzyme-linked immuno-sorbence assay (ELISA). In a tissue culture plate having six wells, the residual medium is rinsed out using 0.5 ml of trypsin/EDTA solution (37°C; 0.025% of trypsin) per well. The cells are then detached from the bottom using 1 ml of trypsin/EDTA per well. The trypsin should not remain on the cells for longer than five to eight minutes in order to

avoid irreversible damage. After the cells have been detached from the bottom, neutralization is carried out using 1 ml of serum-containing culture medium 2 (prewarmed). The cell suspension is collected in a centrifuge tube with a screw closure and centrifuged at about 300 g and 4°C for 10 minutes. The supernatant is aspirated and the cell pellet is suspended using 1 ml of liquid or viscous solution and counted.

The liquid or viscous cell suspension is drawn into an aspiration chamber in the three-dimensional carrier materials by reduced pressure without loss of volume such that the entire inner surface is populated.

b) Ex vivo culture of the constructs

The cell-biomaterial constructs are brought into a special sterile perfusion chamber and expanded such that a unidirectional flow of the culture medium is guaranteed. In addition to the supply of always fresh medium, the cells are stimulated mechanically by the liquid flow. The culture medium (osteoblast medium, e.g. BGJ-B medium, Gibco, with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 10% (v/v) of serum; in the case of therapeutic use autologous human serum is used, in the case of experiments fetal calf serum) is enriched by addition of 10^{-8} to 10^{-10} M dexamethasone, 50 µg/ml of vitamin C and 40 ng/ml of vitamin D3 in the supplying part in order to stimulate the cells to matrix synthesis.

c) Analysis of the constructs in the culture phase

For the measurement of the cell metabolism, a tetrazolium salt "XTT" is added to the constructs. The test is based on the colorimetric biotransformation of the salt to the intensively yellow-colored formazan in the mitochondria. The formazan concentration is measured photometrically at a wavelength of 450 nm at specified points in time after the addition of the solution. The result correlates with the cell metabolism and the

proliferation of the cells (cell proliferation kit, Roche Diagnostics, Mannheim).

The adhesion can be checked by "environmental scanning electron microscopy" (ESEM). "Environmental scanning electron microscopy" is a technical process of electron microscopy, in which cells do not have to be fixed, but can be inspected living in culture medium by electron microscopy. 48 hours after population of the biomaterials, the constructs are adhered to a carrier and transferred to an ESEM chamber (Freiburger Materialforschungsinstitut). The adhesion of the cells to the material surface can be shown hereby in the magnification of an electron microscope.

d) Analysis of the constructs at the end of the culture phase

After two to four weeks in the perfusion chamber, the constructs are fixed and investigated histologically for new matrix formation on the carrier materials by the classical HE staining. New bone tissue is stained intensively blue by the special Richardson-Levaletzko stain.

By means of immunohistochemistry, collagen 1 is detected as an organic main constituent of the bone tissue (e.g. by the avidin-biotin method).

By means of immunofluorescence using FITC-labeled antibodies, osteocalcin is demonstrated as a specific bone marker in the new tissue.

e) Addition of angiogenic growth factors to the bone component (optional)

For the induction of the new vessel formation, growth factor proteins (VEGF, bFGF, Ang I or Ang II) are added to the bone component. Firstly, an osteoblast cell culture is established as described in example 2. The subconfluent cells in a 75 cm^2 cell

culture flask are trypsinized for five minutes using 1 ml of 0.025% trypsin/EDTA solution. After resuspension in 2 ml of medium using 10% FCS and centrifugation at 1000 rpm and 4°C for 5 minutes, the cells are resuspended in 100 µl of medium and counted. 20,000 osteoblasts from the cell passage 1 to 3 are suspended in 200 µl of fibrinogen solution (66 mg of fibrinogen dissolved in 1 ml of αMEM or medium 199 or BGJ-B medium without serum with 100 U/ml of penicillin, 100 mg/ml of streptomycin; 0.1 to 10% of ε-amino-n-caproic acid). 40 µl of a 40 mM calcium chloride solution containing 1.25 I.U. of thrombin/ml and 0.1 to 10 ng/ml of recombinant growth factor are then added to the osteoblast-fibrinogen suspension. The mixture is then injected into a cell culture dish (for example 48 well plate). After addition of 760 ml of culture medium BGJ-B with 10% FCS and 100 U/ml of penicillin and 100 mg/ml of streptomycin, the cell construct is cultured in a warming cabinet at 37°C, 5% CO_2 and 100% atmospheric humidity.

f) Preparation of an osteoblast-endothelial cell-fibrinogen suspension OEFS (optional)

An osteoblast cell culture is established as described in example 2. The subconfluent osteoblast culture in a 75 cm^2 cell culture flask is trypsinized for 5 minutes using 1 ml of 0.025% trypsin/EDTA solution. After resuspension in 2 ml of endothelial cell medium without serum and centrifugation at 1000 rpm and 4°C for 5 minutes, the cells are resuspended in 100 µl of endothelial cell medium and counted. 1 to 10 x 10^4 (preferably 20,000) osteoblasts from the cell passage 1 to 3 are suspended in 200 µl of fibrinogen solution (66 mg of fibrinogen in 1 ml of endothelial cell medium with 100 U/ml of penicillin, 100 mg/ml of streptomycin and 0.1 to 10% of ε-amino-n-caproic acid). In parallel to this, an endothelial cell culture is established according to standard protocol as a microvascular cell culture. The subconfluent endothelial cell culture in a 75 cm^2 culture flask is trypsinized for 5 minutes using 1 ml of 0.025% trypsin/EDTA solution. After resuspension of the cells, 2 ml of endothelial cell medium without serum and centrifugation for 5 minutes at 1000 rpm and 4°C, the

cells are resuspended in 100 µl of endothelial cell medium and counted. 1 to 10 x 10^4 (preferably 20,000) endothelial cells from cell passage 1 to 3 are suspended in 200 µl of the above-mentioned fibrinogen solution. Finally, the osteoblast-fibrinogen suspension and the endothelial cell-fibrinogen suspension are mixed with one another by resuspension. The osteoblast-endothelial cell-fibrinogen suspension (OEFS) is aspirated into a porous, three-dimensional carrier material, e.g. autoclaved spongiosa. Per ml of OEFS, 40 µl of calcium chloride-thrombin solution are added (1.25 I.U. of thrombin per ml, 40 mM calcium chloride). The construct is introduced into a culture dish or a perfusion chamber. Endothelial cell medium is then added with 10% FCS, 100 U/ml of penicillin and 100 mg/ml of streptomycin. Culturing is carried out in a warm cabinet at 37°C, 5% CO_2 and 100% atmospheric humidity for 1 to 7 days until fusion with the cartilage component.

By means of addition of the calcium chloride-thrombin solution, a three-dimensional fibrin network with adhering osteoblasts and endothelial cells is formed in the fibrin matrix, which fills the porous carrier material. In the light microscope (100-times magnification), the formation of the osteoblastic phenotype with dendritic cell branches and the construction of intercellular connections of the osteoblasts is seen. In between there are relatively small endothelial cells, which proliferate in vivo and can organize to give capillaries.

The vitality can be investigated by Trypan Blue staining after 7 days. To this end, the supernatant of the culture medium is aspirated, 50 µl of Trypan Blue solution are added, then the preparation is checked under the light microscope. After 7 days, only a few dead cells are found.

Example 6: Preparation of ligament components

Fibroblasts are inoculated onto ligamentous materials as a suspension having 1 x 10^7 to 5 x 10^7 cells/ml of medium and

maintained in culture in Dulbecco's minimal essential medium (DMEM) medium containing 10% autologous serum at 37°C and 5% CO_2.

Example 7: Fusion of the individual components

Primary chondrocyte cultures were established from rib cartilage biopsies and suspended at a density of 2×10^7 cells/ml in the thrombin component of a fibrin adhesive. The fibrinogen component is then mixed together with the chondroblast-containing thrombin component by means of a double syringe and poured into a structured, anatomically appropriate mold. The fibrin adhesive solidifies in the desired shape within 5-10 minutes as a function of the thrombin concentration. In parallel, primary osteoblast cultures are prepared from iliac crest biopsies (see example 2) and the osteoblastic phenotype is detected by alkaline phosphatase and osteocalcin. The cells are applied to three-dimensional, porous biomaterials (e.g. bovine or human spongiosa) as a suspension containing 1×10^6 to 1×10^7 cells/ml of viscous gel or liquid medium by vacuum aspiration. The adhesion of the cells is checked by electron microscopy and the proliferation by XTT metabolic tests. The individual constructs are separately cultured for three days in vitro at 37°C and 5% CO_2. Following this, the fusion of the bone and cartilage component is carried out by means of fibrin adhesion.

The fusion can also be achieved in a modified manner as follows: during the process of the production of the cartilaginous component and the setting of the fibrin adhesive, the empty, preformed and appropriate bone carrier material is lightly pressed into the cartilaginous layer, so that it is firmly bound.

In the second step, the population of the bone component by the osteoblast suspension is then carried out in a viscous gel or liquid medium.

As the result of an experiment of this type, cartilaginous and bone component were firmly fused to one another. The cartilage

surface was smooth and was clearly definable from the bone component. Macroscopically, a correct, stable anatomical joint shape corresponding to the natural anatomical conditions existed.

Cartilage-bone constructs with ligament apparatus:

The cartilage-bone constructs described above are connected with ligament constructs by drilling a channel 0.5 mm in size through the bone component and drawing through the ligament construct, consisting of fibroblasts on a collagen ligament.

The ligament construct is firmly integrated and can connect and hold two cartilage-bone constructs such that the cartilage surfaces articulate and can move against one another.

Animal experiments:

Nude mice about six to eight weeks old were anesthetized in an anesthesia chamber containing an ISOFLURANE®-oxygen mixture (3% ISOFLURANE® in 100% O_2, flow 4 l/min). While maintaining the anesthesia using an inhalation mask (1.5 - 2% (v/v) ISOFLURANE® in 100% O_2, flow 0.5 to 1 l/min) the animals were washed with BETAISODONA® and the operation area was sterilely covered. A skin incision about 2 cm long running longitudinally was carried out in the region of the back, spreading with the preparation forceps and creating a skin pouch. The osteochondral transplant (about 2 x 0.5 x 0.5 cm) was inserted and the wound was closed using individual button sutures. A sterile wound dressing was applied. The intervention lasted approximately 15 minutes. The wounds of the animals were checked daily until wound healing had been confirmed.

The nude mice finally received a lethal dose of CO_2 by inhalation on the 21st, 42nd or 63rd day postoperatively. The osteochondral constructs were excized with the surrounding tissue and then worked up histologically and immunohistochemically. As a result, the cartilaginous and bone component were firmly connected to one another in the fused osteochondral constructs. Histologically, the

cartilaginous layer showed the morphology of hyaline cartilage tissue with immunohistochemical detection of type 2 collagen. In the bone component, an appositional bone growth with inbranched capillaries was found. In the biomechanical testing, the cartilage component showed a rigidity (compression modulus) of 0.59 Mpa and a hydraulic permeability of 1.03×10^{-14} m^4/Ns in the "confined compression test".

Example 8: Lipofection of osteoblasts with EGF plasmid

Human osteoblasts are distributed on tissue culture containers on the day before transfection. In this case, tissue culture dishes having six wells (Costar, Cat. No. 3506, 9.6 cm^2 per well), in the following called "6-well plates", and cell culture inserts (Falcon Cell Culture Inserts, Becton Dickinson, PET membrane with 1.6 x 10^6 pores (1 µm) per cm^2, Cat. No. 3102, 4.2 cm^2) were used.

Two protocols follow with which high expressions are achieved.

a) Transfection of subconfluent monolayer cultures in 6-well plates

For the preparation of the transfection solution, 230 µl of medium 1 without additives, 15 µl of ESCORT® reagent (Sigma) and 6 µg of plasmid DNA (comprising the gene for human EGF) are mixed per batch. After incubation at room temperature for 15 min, 2 ml of medium 2 are added to the solution per batch. The culture medium is removed from the cells and the transfection mixture is added to the cells (2.5 ml/well). After this, the cells are incubated in an incubator at 37°C and 5% CO_2 for 12 h. The transfection mixture is removed from the cells, which are then washed with medium 2. Finally, for further culturing about 3 ml of complete medium are added to each well. The medium is changed daily, samples of the supernatants are stored at -20°C, which are then used for the determination of the EGF content. The epidermal growth factor (EGF) concentration was determined by means of an ELISA (QUANTIKINE™, R&D Systems, Cat. No. DEG-00). For this, the samples must optionally be diluted 1:10-1:15 in medium beforehand, since the measuring range of the ELISA is 0-250 pg/ml.

The analysis showed that EGF was secreted over 10 days with a maximum on the 5th day.

b) Transfection of subconfluent monolayer cultures in cell culture inserts ("separating chamber experiment")

The cell culture inserts are inserts which have a porous membrane, to which the cells can adhere. The insert is employed in a tissue culture plate having (usually 6) wells, so that the "apical" cells in the insert are about 3 mm spatially separate from the "basal" cells in the well of the culture dish, but can exchange signal substances via the permeable membrane by diffusion. Medium is added to the "basal" and "apical" side of the cells, that is in the well of the cell culture dish in which the insert is employed and in the insert itself. This arrangement can be used in order to determine a paracrine effect of secreted growth factors on other cells. To this end, osteoblasts are inoculated into the cell culture insert and are transfected. After the transfection, the inserts are transferred to plates which are inoculated with nontransfected cells. Since the medium and factors which are secreted by the transfected cells can pass through the filter, an increased proliferation can be detected by cell count determination of the nontransfected cells in the other compartment.

The transfection solution is prepared as in a). The culture medium is removed from the well of the culture plate and from the insert as completely as possible. 2.25 ml of transfection solution are added to each insert. After incubation in an incubator for 30-60 min, 1 ml of complete medium is added. If, during the incubation phase, the transfection solution rapidly runs through the filter of the insert, medium must optionally be added earlier. Incubation is then carried out at 37°C and 5% CO_2 for 12 h. The transfection solution is filtered off with suction and the insert is washed copiously with medium. The insert can now be transferred to a culture dish containing cells. The volume of medium is a total of 4 ml/well and insert.

In one experiment, 10 inserts inoculated with osteoblasts were transfected with EGF plasmid. As controls, 10 inserts containing

cells only were treated with liposome solution without plasmid, a further 6 inserts were left completely untreated, a further 6 inserts were transfected with EGF plasmid, after the transfection, however, 11.9 µg/ml of medium-neutralizing anti-EGF antibody was added daily (R&D, No. AB-236-NA, 1 mg/ml in the PBS, pH 7.4, sterile). On day 6 after the transfection, the cell count of the nontransfected cells in the lower compartment was determined by cell counting using a "Casy TT" cell counter. Using the apparatus manufactured by Casy, cells in solutions can be counted spectrometrically. The result is shown in the figure 2. Both control batches ("liposome" and "untreated") show significantly lower cell counts than the batch in which EGF plasmid was transfected ("EGF/liposome"). This shows that transfected cells have a proliferative effect. The third control ("antibody") in turn shows lower cell counts than the batch with transfected cells, but without antibody ("EGF/liposome"). This proves that the proliferative effect is to be attributed to EGF. This experiment shows that, by means of transient lipofection of osteoblasts, cells can be produced which secrete growth factors which can stimulate the growth of other cells.

In a further "separating chamber experiment", the proliferative effect of EGF-transfected osteoblasts was compared with the proliferative effects of recombinant, human EGF, which was added to the cells daily. On day 4 after transfection, the cell count of the various batches was determined. This was carried out by automatic cell counting by means of "Casy TT" and by counting out in a Neubauer counting chamber. The result is shown in figure 3. After transfection with EGF-DNA ("EGF transfection"), the cell count of the transfection batch is almost twice as high as that of the nontransfected control batch ("control"). Added recombinant EGF (1 ng/ml each day) in fact also causes increased growth ("rhEGF addition"), but not as strong as is achieved by the transfection.

Example 9: Lipofection of osteoblasts with hbFGF plasmid

Osteoblasts are transfected with a plasmid which contains a nucleic acid which codes for human "basic fibroblast growth factor" (hbFGf).

On the day before the transfection, the cells are distributed on appropriate culture containers (e.g. 6-well plates or cell culture inserts for the separating chamber experiment). 40,000 cells each are employed per well of the 6-well plate (9.6 cm^2 growth area per well) or per cell culture insert (4.2 cm^2 growth area per insert). Two protocols follow with which a high expression of the transfected genes was achieved.

a) Transfection of subconfluent monolayer cultures in 6-well plates (ESCORT™ -reagent, Sigma)

The transfection solution is prepared by mixing 115 µl of medium 1, 9 µl of ESCORT™ reagent (Sigma) and 3 µg of hbFGF-plasmid per batch. Alternatively, 115 µl of medium 1, 15 µl of ESCORT™ reagent and 5 µg of plasmid are mixed. After incubation at room temperature for 15 min, the culture medium is removed from the cells, instead of this 1 ml of medium is added to the cells with additives, followed by the transfection solution. Incubation is then carried out at 37°C and 5% CO_2 for 1-2 h. Addition of 2 ml of medium/well and incubation at 7°C and 5% CO_2 for 24 h follows. The medium is furthermore changed daily, samples of the culture supernatant being stored at -20°C until analysis. The bFGF concentration in the samples is determined by ELISA. The result is shown in figure 4. When using 3 µg of DNA and 9 µl of ESCORT™, the FGF secretion on day 3 after transfection is highest, when using 5 µg of DNA and 50 µl of ESCORT™ it is highest on day 5 after transfection. The amount of FGF given off into the medium is higher during transfection with 5 µg of DNA.

b) Transfection of subconfluent monolayer cultures in 6-well plates (FUGENE® reagent)

The transfection solution is prepared by mixing of medium without additives, FUGENE® [®] reagent (Roche Diagnostics) and DNA. Three variants were tested:

Batch:	A	B	C
µl of medium without additives:	97	94	91
µl of FUGENE® reagent	3	6	9
µg of hbFGF plasmid	3	3	3

Here, the medium and the FUGENE® reagent in each case are first mixed and incubated at room temperature for 5 min. The DNA is then added, and after mixing incubation is carried out at room temperature for a further 15 min. The medium is removed from the cells, then 3 ml of medium and afterwards the transfection solution are added per well. Incubation at 37°C and 5% CO_2 for 24 hours in an incubator follows. In the following, the medium is changed daily, samples of the culture supernatant in turn being stored at -20°C until analysis. The FGF concentration in the medium is determined by ELISA. The amount of DNA can also be varied when using the FUGENE® reagent. 3 and 5 µg of plasmid were tested. The result is shown in figure 5. On day 7 after the transfection, when using 3 µg of DNA more FGF is secreted than when using 5 µg of DNA ("3 µg of plasmid/FUGENE® and 5 µg of plasmid/FUGENE®"). As a control, only DNA without FUGENE® reagent was also "transfected". The measured amount of FGF is clearly lower than when using FUGENE® ("3 µg of plasmid" and "5 µg of plasmid"). As a further control, the fibroblast growth factor (FGF) concentration in the medium from completely untreated cells ("untr. cells") was determined.

The result shows that osteoblasts can be transfected successfully with hbFGF-DNA and that as a result more FGF is given off to the medium.

a) Transfection of subconfluent monolayer cultures in cell culture inserts ("separating chamber experiment")

The transfection solution is prepared by mixing 115 µl of medium without additives, 15 µl of ESCORT™ reagent and 5 µg of hbFGF plasmid. After 15 min at room temperature, 2 ml of medium 2 are added. The culture medium is removed as completely as possible from the cell culture insert in which the cells to be transfected are present, and from the well of the 6-well plate in which the insert is located. 2.25 ml of transfection solution are added to the cells per insert. Incubation is carried out for 30-60 min at 37°C and 5% CO_2 in an incubator. 1 ml of complete medium per insert is then added (where appropriate further medium must be added if the transfection solution runs through the filter of the insert rapidly). Incubation is again carried out for 24 h at 37°C and 5% CO_2 in an incubator. The transfection solution is then filtered off with suction, and the insert is copiously washed with medium. The insert is now employed in a culture dish containing nontransfected cells whose proliferation is monitored. The medium volume of insert and culture dish together is 4 ml.

On day 4 after the transfection, the cell count of the nontransfected cells was determined in the lower compartment in such an experiment. Figure 6 shows the result. The control shows the natural proliferation of cells in 4 days ("untr. cells"). Both the presence of hbFGF-transfected cells ("5 µg of plasmid/15 µl of ESCORT™ and addition of recombinant human bFGF ("rec. bFGF 4 ng/ml") lead to increased proliferation of the cells in comparison with the control. This shows that the transfected cells can paracrinely stimulate the growth of nontransfected cells by FGF secretion.

Example 10: Lipofection of osteoblasts with hVEGF plasmid

Subconfluent osteoblasts are transfected with DNA which codes for human "vascular endothelial growth factor" (hVEGF). On the day before the transfection, the cells are distributed on appropriate culture containers. When using 6-well plates, 50,000 cells per well are employed.

The transfection solution is prepared by mixing medium without additives, FUGENE® reagent (Roche Diagnostics) and DNA. 4 variants were tested:

Batch:	A	B	C	D
µl of medium without additives:	94	91	90	85
µl of FUGENE® reagent	6	9	10	15
µg of hVEGF plasmid	3	3	5	5

In this case, the medium and the FUGENE® reagent are first mixed and incubated at room temperature for 5 min. The DNA is then added, and after mixing incubation is carried out for a further 15 min at room temperature. The medium is removed from the cells, then 3 ml of medium and afterwards the transfection solution are added per well. Incubation for 24 hours at 37°C and 5% CO_2 is carried out in an incubator. In the following, the medium is changed daily, samples of the culture supernatant in turn being stored at -20°C until analysis. The VEGF concentration in the medium is determined by ELISA. The result of the above-mentioned batches is shown in figure 7. On day 7 after the transfection, the VEGF concentration was determined. The 4 batches ("A" to "D") show, compared with the control, which shows the VEGF concentration in the medium of untreated cells ("untr. cells"), increased VEGF values. The values are also higher than in the case of batches in which the cells were only treated with plasmid, but without FUGENE® reagent ("5 µg of plasmid").

The inductive effect of transfected osteoblasts (batch B of the table in example 10) on the growth of microvascular endothelial cells was investigated in the separating chamber (for process see example 8b)). The result is shown in figure 8. A clearly accelerated growth of the endothelial cells compared with the control without transfected osteoblasts was seen.

Example 11: Electroporation of osteoblasts with EGF plasmid

Osteoblasts in culture are detached by trypsinization, centrifuged and counted. The cell suspension is adjusted to 1.5 x 10^6 cells/ml. 300 µl each of this suspension are transferred to an electroporation cuvette (GENE PULSER®/E. coli PULSER™ cuvette, Cat. No. 165-2086, 0.2 cm gap electrode, BioRad, CA 94547). The gap electrode is an electrode with which electric fields are produced in the nutrient medium during the electroporation. 30 µl of DNA (hEGF plasmid) are added to the cells in the cuvette. A control cuvette with cells prepared in parallel contains no DNA. The cuvettes are then incubated on ice for 10 min.

The actual electroporation is carried out using an apparatus for carrying out electroporations "Gene Pulser II System" from BioRad (Cat. No. of the main unit: 165-2105). The settings are 150 or 250 V and 960 µF; the electroporation time is determined automatically by the apparatus, it is approximately 4 seconds. The cuvette is mounted in the holder of the "shocking chamber" (chamber in which cells are introduced in Näher solution and in which electrical fields can be produced), where the electroporation takes place. After the electroporation, the cuvette is removed from the holder and incubated at 4°C for 10 min. The cells are resuspended in medium (osteoblast medium, e.g. BGJ-B medium, Gibco, with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 10% (v/v) of serum; in the case of therapeutic use autologous, human serum is used, in the case of experiments fetal calf serum) and distributed on tissue culture dishes. The medium is changed daily (detection of EGF "noncumulative"). Alternatively, the medium cannot be changed (detection of EGF "cumulative"). In a "noncumulative" experiment, samples were taken daily from the 4 ml of culture supernatant, and were stored at -20°C until the EGF concentration had been determined by ELISA. However, 1.25 x 10^6 human osteoblasts were electroporated (250 V, 960 µF; 3.9 s, 20 µg of DNA). The result is shown in figure 9. The measured EGF concentration is plotted as a function of the time in days. The EGF secretion into the medium has a maximum on day 2 after transfection. EGF expression is detectable for more than one week.